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EphA2 is an Essential Mediator of Ultraviolet Radiation-induced Apoptosis

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Abstract

One of the physiological consequences of excessive ultraviolet radiation (UVR) exposure is apoptosis. This critical response serves to eliminate genetically-injured cells and arises, in part, from activation of DNA damage and p53 signaling. Other contributory pathways, however, likely exist but have not been fully characterized. In a recent global screen of UVR-response genes in melanocytes, we identified the receptor tyrosine kinase (RTK), *EPHA2*. Using a combination of genetic and pharmacological approaches, we set out to investigate the upstream regulation EphA2 by UVR and the functional consequences of this effect. We found that the UVR-associated increase in EphA2 occurs in melanocytes, keratinocytes and fibroblasts from both human and murine sources. More specifically, UVR effectively upregulated EphA2 individually in p53-null, p63-null and p73-null murine embryonic fibroblasts (MEFs) suggesting that the p53 family of transcription factors is not essential for the observed effect. However, inhibition of MAPK signaling by U0126 and PD98059 significantly reduced the UVR response while overexpression of oncogenic *NRAS* led to an increase in EphA2. These results confirm that UVR induces EphA2 by a p53-independent but MAPK-dependent mechanism. In response to UV irradiation, *Epha2*^{-/-} MEFs were highly resistant to UVR-mediated cytotoxicity and apoptosis while introduction of EphA2 into both wildtype and p53-null MEFs led to activation of an apoptotic program that can be blocked by caspase-8 inhibition. These functional findings suggest that EphA2 is in fact an essential p53-independent, caspase-8 dependent pro-apoptotic factor induced by UVR.

INTRODUCTION

The orchestrated genetic and biochemical response of various cells to ultraviolet radiation (UVR) is highly complex and is still largely unresolved. Much of the intracellular signaling

and transcriptional events are aimed at defending the genome against mutagenic stress brought about by excessive UVR. In addition, reactive oxygen species (ROS) contributes to cellular stress and triggers intracellular signaling and DNA damage(1). The p53 transcription factor has drawn particular attention given its central role in DNA damage and apoptotic signaling. However, UVR-induced apoptosis is highly complex and appears to occur through both p53 dependent or independent pathways(2).

In a recent effort to catalogue all UV-mediated transcriptional events in melanocytes, we identified a p53 transcriptional target, *EPHA2*, that is significantly upregulated by UVR(3). EphA2 belongs to the Eph receptor tyrosine kinase family, which is the largest group of tyrosine kinases in the genome(4). The Eph receptors mediate cell-cell signaling through glycosylphosphatidylinositol (GPI) lipid-anchored ligands (ephrin A) or transmembrane ligands (ephrin-B). Since high EphA2 levels have been reported in melanomas(5, 6) and have been associated with a more malignant phenotype(7), the induction of EphA2 by UVR exposure provides a possible mechanistic link between excessive UV exposure and melanoma risk. Moreover, a large body of evidence suggest that EphA2 is overexpressed in many cancer types(8) thereby raising the possibility that EphA2 controls basic cellular processes that are not limited to pigment cells. We thus set out to further characterize the pathway(s) responsible for the upregulation of EphA2 by UVR and the possible role that this gene plays in UV photobiology.

MATERIALS AND METHODS

Antibodies and Inhibitors

Antibodies used in this study were as follows: polyclonal anti-EphA2(C-20), monoclonal anti- β -tubulin (D-10), polyclonal anti-p63 and polyclonal anti-p73 (Santa Cruz; Santa Cruz, CA); monoclonal anti-GAPDH (Abcam; Cambridge, MA); polyclonal anti-p53(CM5; Novocastra Laboratories Ltd; Newcastle upon Tyne, UK); polyclonal anti-ERK, anti-phosphoERK(Thr202/204) and rabbit monoclonal anti-poly(ADP-ribose) polymerase (PARP; Cell Signaling Technology; Beverly, MA); goat anti-mouse conjugated HRP(Bio-Rad; Hercules, CA); goat anti-rabbit IgG-HRP(A1905) (Santa Cruz; Santa Cruz, CA).

Pharmacological inhibitors of MEK (U0126, PD98059), p38 MAP kinase inhibitor (2-(4-Chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1,2-dihydropyrazol-3-one; Cat No 506126), as well as JNK inhibitor (JNK Inhibitor II; SP600125; Cat No 420119) were all purchased from Calbiochem (San Diego, CA). Phosphatidylinositol-3-kinase Inhibitor (LY294002) was purchased from Cell Signaling Technology, Inc (Danvers, MA). Both Caspase-8 (catalog no 218773) and caspase-9 inhibitors (catalog no 218728) were from CalBiochem (San Diego, CA).

Cell culture and ultraviolet irradiation

Normal human melanocytes (NHMs) were purchased from Cascade Biologics(Portland, OR) while immortalized NHMs and immortalized normal human keratinocytes (NHKs) (both engineered through the successive introduction of p53DD, CDK4^{R24C} and hTERT) were kindly provided by David Fisher (Dana Farber Cancer Institute; Boston, MA) and

James Rheinwald (Brigham and Women's Hospital; Boston, MA), respectively. Human melanoma cells lines have been previously published(9). Murine embryonic fibroblasts (MEFs) from *Trp53*, *Trp63* and *Trp73* deficient mice were kindly provided by Elsa R. Flores (M.D. Anderson Cancer Center; Houston, TX) while MEFs from *Epha2*^{-/-} mice(10) were isolated using standard protocols.

The NHMs and immortalized NHMs were maintained in Medium-254 (Cascade Biologics; Portland, OR) supplemented with human melanocyte growth supplement and 1% penicillin/streptomycin (1% P/S). Melanoma cell lines and MEFs were maintained in DMEM supplemented with 10% fetal bovine serum (Sigma-Aldrich; St. Louis, MO) containing 1% P/S. Immortalized NHKs were cultured in keratinocyte serum-free medium (GIBCO ker-sfm)(GIBCO/Invitrogen; Grand Island, NY) supplemented with bovine pituitary extract (25µg/ml;GIBCO/Invitrogen;Grand Island, NY), EGF (0.2ng/ml;GIBCO/Invitrogen;Grand Island, NY), and CaCl₂ (0.4mM;Sigma, St. Louis, MO).

Ultraviolet irradiation and treatment with inhibitors

We have previously described our UVB irradiation protocol (3). For inhibition studies, cells were plated the day before exposure and pre-treated with molecular inhibitors targeting MEK, p38 MAP kinase, JNK or PI-3 kinase for 1 hr prior to irradiation. After UVB exposure, cells were then dislodged into 1× DPBS, centrifuged at 1500 rpm for 5 minutes at 4°C, lysed and frozen at -80°C until immunoblotting.

Immunoblot analysis

SDS gel electrophoresis (SDS-PAGE) was performed according to standard protocols. Ten µg of total cell lysate were diluted in 6× Laemmli buffer (Boston Bioproducts Inc; Worcester, MA) and loaded onto a 10%-precast gels (Bio-Rad; Hercules, CA), transferred onto Immobilon-P membrane (Millipore Corporation; Billerica, MA) and blocked with 5% nonfat milk (Bio-Rad; Hercules, CA) in Tween (1%)/TBS. Primary antibody dilutions used in this study were as follows: polyclonal anti-EphA2(1:300), polyclonal anti-ERK (1:800), polyclonal anti-phosphoERK (1:200), polyclonal anti-p53, anti-p63 or anti-p73 (all 1:500). Loading equivalence was monitored with monoclonal anti-β-tubulin (1:150) or monoclonal anti-GADPH (1:3500) and gels were visualized with the ECL system (Amersham Bioscience; Piscataway, NJ) after application of 1:5000 goat anti-rabbit-HRP (1:5000).

Plasmid construction and transfection

To generate the eukaryotic expression plasmids encoding wildtype *NRAS* and *NRAS*^{Q61R}, *NRAS* cDNAs were PCR cloned from the melanoma lines A375 and Roth(9), respectively. A donor human *EPHA2* cDNA plasmid (Harvard Institute of Proteomics; Cambridge, MA) was transferred into the pLP-IRESneo acceptor using the Creator™ kit (Clontech; Mountain View, CA) per manufacturer's protocol. All plasmids were confirmed by DNA sequencing and immunoblotting after either transfection with FuGENE-6 (Roche Diagnostics; Indianapolis, IN) or Nucleofection (Amaxa; Gaithersburg, MD).

Apoptosis assays

For both UVB-associated and EphA2-mediated apoptosis, we plated 100,000 cells in 60mm dishes about 21 hours prior to either UVB irradiation (80 mJ/cm² UVB) or transfection (5 µg pLP-IRES-EphA2 or pLP-IRES vectors). Treated cells were analyzed for intracellular casapase-3 activity at 24 hours (UVB irradiation) or 30 hrs (EphA2 transfection) using the Caspase-3-Activity Detection Kit (Upstate; Lake Placid, NY).

For propidium iodide (PI) staining, 800,000 cells were harvested and then fixed by 70% ice-cold EtOH overnight. After fixation, cells were washed with PBS and then stained with 100 µg/ml PI in PBS containing 100 µg/ml RNase A and 0.1% NP40 (all from Sigma; St Louis, MO). PI-stained cells were analysis by FACSCalibur (Becton Dickinson; San Jose, CA).

RESULTS

UVR-mediated upregulation of EphA2

In order to determine if UVR selectively induces EphA2 among other members of the Eph/ephrin family, we re-analyzed our microarray data(3) to assess the effect of UVR exposure on other ephrins and Eph receptors. As shown in supplemental Figure S1, EphA2 stands out among other Eph/ephrin genes in both level of stimulation and significance of increase. There is no apparent predilection for either Ephrins or Eph receptors, as a group, to be induced. Thus, EphA2, among its relatives, is selectively upregulated by UVR.

Our previous data demonstrated an increase in EphA2 mRNA levels in response to UVR(3). Figure 1A shows that EphA2 protein levels are induced by UVR within 5 hours and reach a maximum by approximately 12 hours post-irradiation in normal human melanocytes (NHMs); this level is sustained for at least 48 hours (data not shown). There is also a dose-dependent increase in EphA2 levels starting at about 15mJ/cm² UVB (Figure 1B) and reaching a maximum around 35-50mJ/cm².

Since there are extant reports that EphA2 can be transcriptionally induced by p53, we used two complementary strategies to determine if the UVR-mediated upregulation of EphA2 is dependent on p53 in melanocytes. First, we exposed NHMs that have been immortalized with p53DD/CDK4^{R24C}/hTERT to UVR and found a similar level of EphA2 induction as the non-immortalized NHMs (Figure 1C). Next, we irradiated a panel of melanoma cell lines and found that lines with homozygous deleterious p53 mutations (WM164, p.Tyr220Cys; MM-LH, p.Gly244Arg) were still able to upregulate EphA2 in response to UVR (Figure 1C). These initial results provide suggestive evidence that primary immortalized and transformed human melanocytes do not require p53 for the observed UVR EphA2 effect.

Although we initially identified this EphA2 regulatory event in melanocytic systems, all cells maintain the capacity to counter extracellular stress and thus we hypothesized that this novel UVR response may be preserved in cells of different origins. We subjected immortalized p53-inactivated normal human keratinocytes (ie. by introduction of p53DD/CDK4^{R24C}/hTERT) to UVR and found a similar level of EphA2 induction as in melanocytes (Figure 1C). This is significant as keratinocytes represent the most direct target of both UVR stress and possible p53 mutagenesis(11).

Additionally, murine embryonic fibroblasts (MEFs) represent a highly tractable system to monitor physiological stress in a definable genetic context. As shown in Figure 1D, irradiation of wildtype MEFs, NIH-3T3 cells and *Cdkn2a*^{-/-} MEFs all reproducibly resulted in EphA2 increases. The ability to introduce MEFs into the analysis also allowed us to more definitively exclude the p53 family of transcription factors. To this end, we subjected MEFs that were null for *Trp53*, *Trp63* and *Trp73* to UVR and found that EphA2 protein levels were in fact appropriately induced in all of these MEFs (Figure 1D). Taken together, our results using immortalized melanocytes, p53 and ARF-inactivated melanoma cells and genetically-defined MEFs all strongly support the contention that p53, p63 and p73 are not individually necessary for the observed EphA2 regulation although we cannot eliminate the possibility that there is functional compensation between the family members. It is worth mentioning, however, that these results do not contradict earlier findings that EphA2 is a p53 target gene(12); in fact our data provide evidence for a higher order of complexity in EphA2's response to cellular UVR stress.

Beyond regulation by the p53 family of transcription factors, recent studies have found that EphA2 is also a direct transcriptional target of the Ras/Raf/MAPK signaling(13); thus, we surmised that this cascade may be an alternative mechanism to increase EphA2. In order to specifically eliminate all potential contributions from p53, we carried out our studies using MEFs lacking p53 function (*Cdkn2a*^{-/-} or *Trp53*^{-/-}). As shown in Figure 2A, pre-incubation of MEFs with MEK inhibitors PD98059 (Figure 2A) or increasing concentrations of U0126 (Figure 2B) dramatically reduced the UVR-mediated increases in EphA2. Exposure of cells to p38MAPK and JNK inhibitors did not show the same inhibitory effect (Figure 2C). Interestingly, we did observe, on occasion, higher EphA2 induction when p38MAPK or JNK were inhibited thereby pointing to possible counter-regulation within the UVR stress-activated MAPK response.

To further corroborate that Ras/MAPK signaling is involved in EphA2 regulation, we ectopically expressed oncogenic *NRAS* (*Nras*^{Q61R}, Figure 2D) in normal human melanocytes and indeed reproduced the increase in EphA2. One can thus conclude that the UVR-mediated stimulation of MAPK signaling is the mechanism responsible, at least in part, for the observed EphA2 induction.

Taken together, we show for the first time that a cancer-associated RTK, EphA2, is upregulated by UVR in a p53-independent but MAPK-dependent fashion. This finding is provocative given the high frequency of both oncogene-associated activation of MAPK signaling and the mutational inactivation of p53 in cancer. Thus, to begin understanding the functional consequences of this increase in EphA2 by UVR, we set out to assess a common physiology shared by UVR exposure and cancer- cell survival.

UVR-induced apoptosis is dependent on EphA2

We undertook a genetic approach to abrogate the EphA2 response. More specifically, we exposed both wildtype and primary non-immortalized MEFs from *Epha2*^{-/-} mice to UVR and determined the effect on cell survival. As expected, there was a complete absence of the UVR-mediated EphA2 induction in the *Epha2*^{-/-} MEFs (data not shown). Strikingly, the *Epha2*-null primary MEFs showed substantial resistance to UVR-mediated cytotoxicity

(Figure 3A). This observation lends itself to the hypothesis that EphA2 is in fact an essential modulator of UVR-associated apoptosis. We found that primary *Epha2*-null MEFs exposed to UVB (80mJ/cm²) exhibited a significantly reduced level of caspase-3 activity and DNA fragmentation (ie. sub G1 fraction) compared to wildtype MEFs (Figures 3B, 3C). The *Epha2*^{-/-} MEFs were nevertheless vulnerable to adriamycin-induced apoptosis (data not shown).

EphA2 induces apoptosis via caspase-8 dependent signaling

One explanation for the observed UVR findings is that upregulation of EphA2 directly induces apoptosis. To test this possibility, we overexpressed EphA2 in p53-null human melanocytes (ie. immortalized NHMs, iNHMs), WT MEFs and p53-null MEFs and observed strong increases in caspase-3 activity (Figure 4A) in all three cell types and cleavage of PARP in the melanocytes (Figure 4B). It thus appears that p53 is not essential for either the induction of EphA2 by UVR or the execution of the EphA2-associated apoptotic program. To further elucidate whether EphA2 signals through the intrinsic or extrinsic pathway, we treated iNHM with specific caspase-8 and caspase-9 inhibitors (both at 20μM). As shown in Figure 4C, there is selective abrogation of the EphA2-mediated PARP cleavage when caspase-8 activity is inhibited suggesting that EphA2 engages the extrinsic pathway to execute its death program.

DISCUSSION

In summary, the major finding of our present study is that EphA2 appears to be an essential p53-independent, but MEK-dependent, mediator of UVR-induced apoptosis. The results support a model (Figure 4D) whereby UVR exposure activates the Ras/MAPK signaling cascade which in turn leads to an increase in EphA2 levels and a triggering of the apoptotic program. Since p53 is known to be both induced by UVR and a transcriptional regulator of EphA2(12, 14), it is possible that both p53 and Ras/MAPK signals resulting from UVR exposure converges on EphA2. From our data, it appears that downstream triggering of apoptosis by EphA2 is independent of p53 but critically dependent on caspase-8- a component of the extrinsic pathway. It is not possible to fully exclude participation by the intrinsic pathway as there may be distinct triggering mechanisms in different cell types; studies to uncover these mechanistic details are currently underway.

Our findings relating EphA2 to UVR are consonant with emerging data to suggest that increases in EphA2 levels may serve as an adaptive response to various forms of microenvironmental stress. Xu and colleagues recently reported that EphA2 levels were induced by hypertonic stress in murine kidney cells derived from the inner medullary collecting duct(15). Similarly, Baldwin et al. found that renal ischemic-reperfusion injury also led to increased EphA2 levels through both ERK and Src kinases(16). Finally, Li et al. found that colonic carcinoma cells exposed to deoxycholate led to an increase in EphA2 by a MAPK-dependent but p53-independent mechanism. With the availability of specific *Epha2*^{-/-} cells, our studies enrich this collective experience by directly implicating EphA2 in the apoptotic program attendant to UVR stress.

EphA2's role in tumor physiology is well described but highly complex. In epidermal keratinocytes, EphA2 appears to harbor tumor suppressive effects. Normal epidermal keratinocytes express high levels of both EphA1 and EphA2 (5, 17) but sequentially lose expression of EphA1 during squamous cell carcinoma (SCC) development. Moreover, Guo et al. recent showed that mice deficient for *Epha2* exhibit a greater susceptibility to SCC tumorigenesis compared to normal mice in the 7,12-dimethylbenz(a)anthracene/12-*O*-tetradecanoylphorbol-13-acetate (DMBA/TPA) skin carcinogenesis model (18). These findings in keratinocytic neoplasms stand in sharp contrast to other cancers such as melanomas, breast cancer, prostate cancer, non-small cell lung cancer and colon cancers where EphA2 has been shown to be frequently overexpressed (8) suggesting a more oncogenic role. In melanoma cells, for instance, high EphA2 expression correlates with a more aggressive phenotype characterized by “vasculogenic mimicry” (6, 19-21); this vasculogenic phenomenon is consistent with EphA2's known role in tumor angiogenesis (22, 23). Moreover, high levels of EphA2 are also associated with worse prognoses in ovarian cancers (24, 25), renal cell carcinomas (26) and esophageal SCCs (27). Taken together, these findings have led to some early studies suggesting that EphA2 may be a viable therapeutic target in cancer (22, 28).

The balance between EphA2's oncogenic and apoptotic effects likely depends upon the cellular context and the unique pathways that are activated in response to various microenvironmental stressors. This has been observed with other canonical oncogenes including c-Myc (29) and Ras (30). Clearly, precise regulation of the pro-tumorigenic and pro-apoptotic signals is critical for the life and death decisions confronting normal cells under stress and transformed cells under selection. Although the mechanisms that explain EphA2's seemingly paradoxical effects are unknown, there are some hypothetical models. In a two-signal model, EphA2 may induce apoptosis and proliferation/survival signals through distinct pathways; in cancers, the pro-apoptotic pathway may be abrogated by concomitant mutations. Alternatively, in a gain-of-resistance model, cells that are selected to survive in the face of escalating death stimuli, including EphA2, may become genetically more aggressive. Additional investigations are clearly needed to fully appreciate the cellular and genetic contexts that specify EphA2 function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. UV-mediated induction of EphA2 is cell autonomous and p53-independent
 EphA2 induction by UVR in NHMs is time (A; 0, 0.25, 0.5, 2, 9, 12 and 24 hrs) and dose (B; 0, 15, 25, 35, 50 mJ/cm²) dependent. The upregulation of EphA2 is also observed in primary human melanocytes immortalized with p53DD, CDK4R24C and hTERT (data not shown). Since immortalized NHMs have ectopically abrogated p53 function (by p53DD), we also set out to determine if melanoma lines were UVR-responsive. (C) NHMs and immortalized NHMs and NHKs (p53 inactivated by p53DD construct) all show induction of EphA2 by UVR. In the lower bar graph, 8 melanoma cell lines (Lane 1, SK-Mel 28; Lane 2, SK Mel-119; Lane 3, WM35; Lane 4, MM455; Lane 5, A375; Lane 6, UACC903; Lane 7, MM-L; Lane 8, WM164) were also subjected to UVB irradiation and exhibited a range of EphA2 inducible levels. Since the two cell lines with the greatest degree of upregulation (lanes 7 and 8) both harbor homozygous p53 point mutations, these data suggest that intact p53 function is not needed for EphA2 induction in melanocytic systems. Although not directly mutated, p53 is presumed to be functionally crippled in cell lines 2-6 where ARF is deleted (Δ ARF). Similar results were obtained with primary human keratinocytes (D). We irradiated NIH-3T3 cells, WT and *Cdkn2a*^{-/-} murine embryonic fibroblasts (MEFs) and found that the EphA2 response was preserved in these cells. In order to determine if the p53

family of transcription factors is required for this observed effect, we subjected MEFs deficient in p53, p63 and p73 to UVB and found that EphA2 is appropriately elevated in these lines.

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Figure 2. EphA2 induction by UVR is dependent on MAPK signaling

Cells were treated with MEK inhibitors (A) PD98059 (50 μ M) or (B) U0126 (dosed 3, 8 or 14 μ M) for 1 hr prior to UVB exposure (40mJ/cm²); EphA2 levels were determined 24 after irradiation. The relative level of EphA2 in unirradiated cells is normalized to 1.0. There is a significant reduction in UVR-mediated upregulation of EphA2 although it is not always complete. (C) The induction of EphA2 at 24 hrs by UVB is not inhibited by 3.5 μ M of THE p38MAPK inhibitor, 5.7 μ M of JNK inhibitor II or 10 μ M of LY294002 (a PI3-K inhibitor). (D) EphA2 can be induced by an oncogenic Nras (NrasQ61R, N*) but not by wildtype Nras (N) or vector (V).

Figure 3. *Epha2*^{-/-} MEFs are more resistant to UV-induced cytotoxicity and UV-mediated apoptosis

(A). WT and *Epha2*-deficient MEFs were exposed to increasing doses of UVB and cell survival was measured at 24 hours. *Epha2*^{-/-} MEFs were clearly more resistant to UVR cytotoxicity. The apoptotic response to UVR (as measured by caspase-3 activity level (B) and DNA fragmentation (C)) was preserved in wildtype MEFs but dramatically reduced in *Epha2*^{-/-} MEFs. *Epha2*^{-/-} MEFs exposed to adriamycin exhibited normal apoptosis (data not shown).

Figure 4. Ectopic overexpression of EphA2 induces apoptosis in a p53-independent, caspase-8-dependent manner

(A). Since loss of EphA2 compromises UVR-mediated apoptosis, we set out to determine if overexpression of EphA2 can induce apoptosis. Ectopic expression of EphA2 led to an increase in apoptosis in immortalized normal human melanocytes (NHMs; inactivated by dominant negative p53DD), wildtype MEFs and p53^{-/-} MEFs. (B). EphA2 induces cleavage of PARP in immortalized NHMs. These results suggests that EphA2 does not depend on intact p53 to bring about apoptosis. (C) Immortalized NHMs transfected with either vector (V) or EphA2 (E) and treated with specific caspase-8 (C8i) or caspase-9 (C9i) inhibitors. There is selective inhibition of PARP cleavage by EphA2 when caspase-8, but not caspase-9, activity is abrogated suggesting that EphA2 mediated effects depend in part on caspase-8 activity. (D). Model of UV-EphA2 circuitry (see text).